Toxicity of azaarenes: mechanisms and metabolism.
Bleeker, E.A.J.

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Chapter 1

General Introduction

Polycyclic aromatic hydrocarbons (PAHs) are molecules composed of two or more fused aromatic rings, either benzene and/or cyclopentene rings. Apart from on-ring substitutions resulting in e.g. oxygenated, hydroxylated, chlorinated, nitro- and/or amino-PAHs, a wide variety of atoms can be incorporated in the aromatic rings, as for example sulphur atoms and nitrogen atoms (Newsted and Giesy, 1987). Although these heterocyclic PAHs outnumber the non-substituted homocycles (Kuhn and Suflita, 1989), they receive relatively little attention in toxicological studies.

Part of the above mentioned on-ring substitutions are due to chemical transformation and degradation of polycyclic aromatic compounds through a variety of processes. In the aquatic environment, photooxidation, chemical oxidation, and biological transformation by bacteria, fungi, algae and animals are of primary importance. Abiotic PAH transformation has been reviewed by Kochany and Maguire (1994), and also much research has been done on biotransformation by bacteria (Kuhn and Suflita, 1989; Bollag and Kaiser, 1991), fungi (Muncnerova and Augustin, 1994; Sutherland et al., 1998), algae (Warshawsky et al., 1995; Dijkman et al., 1997), and animals, both vertebrates (McMurtrey and Knight, 1984; Warshawsky et al., 1996) and invertebrates (Livingstone and Farrar, 1984; Lee, 1988; ). The role of biotransformation often is to reduce toxicity by enhancing the water solubility of a toxicant and thereby facilitating excretion. In many cases, however, especially in carcinogenicity and mutagenicity studies, metabolites have shown to be more reactive than their parent compounds (e.g. Warshawsky, 1992). Hence, metabolism not only greatly enhances the number of toxic compounds, but may also alter the mode of toxic action.
All organic toxicants, including PAHs, induce narcosis, or baseline toxicity, to some extent. Many studies have shown that narcosis is strongly related to the lipophilicity of the compound, often expressed as the n-octanol-water partition coefficient ($K_{ow}$) (Könemann, 1981; De Voogt et al., 1988; Swartz et al., 1995; Chen et al., 1997; Schultz and Bearden, 1998). However, compound specific modes of action (i.e. other than narcosis) cause deviations from such relationships, as has clearly been demonstrated for closely related compounds such as isomers: although they show little differences in lipophilicity, toxicity of isomers may differ several orders of magnitude (Walton et al., 1983; Wood et al., 1983; Kumar et al., 1989; Kraak et al., 1997b). This observation challenges the use of refined structure-activity relationships.

Apart from narcosis, PAHs can affect different biological endpoints: reproductive effects (e.g. Van Brummelen et al., 1996), genotoxicity, teratogenicity and carcinogenicity have been identified (e.g. Warshawsky, 1992). Most research, however, focuses on only one endpoint. It is obvious that this complicates, if not prevents, the search for relationships between the different types of effect. Attempts have been made to predict chronic toxicity from acute test results (Kenaga, 1982; Mayer, 1990; Länge, 1998; Roex, 1999). Such predictions inherently assume a similar kind of action during both acute and chronic exposure. Hence, deviations from such predictions are usually explained by other, more specific effects that are expressed at lower exposure concentrations during a longer exposure time. Morphological deformities and life cycle effects are manifested only after a long period of time (Chang et al., 1984; Taylor et al., 1993; Postma and Davids, 1995; Van Brummelen et al., 1996). Yet, the majority of ecotoxicological studies concerns acute experiments at relatively high doses: only 155 of a total of 1194 items on PAHs in the AQUIRE toxicological database deal with parameters for growth, development, reproduction, population and/or community effects (http://www.epa.gov/ecotox; July, 1999).

**Objectives**

The highly diverse structure of PAHs urges the need for exploring new aspects of their toxicity. Therefore, the aim of the present thesis was to analyse the different aspects of PAH toxicity by evaluating the different kinds of biological effects that azaarenes (nitrogen heterocycles) can exhibit and to relate
these effects to the structure of the compounds. Due to their similarity in structure, comparison of isomer toxicity is attempted to find key factors determining biological effects. Furthermore, biotransformation products, responsible for some of the toxicity of both homocyclic and heterocyclic PAHs, are analysed in different biological species. Traditionally, adverse effects of PAHs are quantified by determining narcotic effects, i.e. baseline toxicity. In the present thesis, however, genotoxicity, developmental disturbances and life-cycle changes are quantified as well. Subsequently, molecular properties are used to relate the different aspects of azaarene toxicity to their molecular structure. Through a baseline toxicity (narcosis) model, the first step towards a fully computational analysis of azaarene toxicity mechanisms are undertaken.

Figure 1.1. The azaarene acridine (left) and its homocyclic analogue anthracene (right).

Heterocyclic Compounds

Two thirds of the 4 million known organic compounds are heterocyclic (Kuhn and Suflita, 1989). Azaarenes are such a group of heterocycles, containing one nitrogen atom in place of a carbon atom (Fig. 1.1). Apart from their natural origin (e.g. as alkaloids; Kaiser et al., 1996), azaarenes are formed and released into the environment by incomplete combustion of fossil fuels, in spills or effluents of several industrial activities, oil drilling, refining and storage (Kochany and Maguire, 1994) and coal tar distillation (Pereira et al., 1983). Azaarenes are also associated with wood preservation (Pereira et al., 1983; Adams and Giam, 1984) and pesticide use (Kuhn and Suflita, 1989). The emission of PAHs, including azaarenes, to the atmosphere has increased greatly during the last century. Historical records of PAHs in soil and sediment from rural areas and in ice from sites as remote as Greenland document the widespread environmental contamination which has occurred (Jones et al., 1989; Sanders et al., 1993; Kawamura et al., 1994). Blumer et al. (1977), Wakeham (1979), Furlong and Carpenter (1982), and Bleeker et al. (1996) detected elevated azaarene concentrations in marine and freshwater sediments. In addition, Van...
Genderen et al. (1994) and Kozin et al. (1997) demonstrated the occurrence of azaarenes in freshwaters in the Netherlands.

For the present study, a group of eleven azaarenes were selected, ranging from 2 to 4-ringed structures, including metabolites and isomers (Fig. 1.2). 5-ringed and larger compounds were not chosen, because their low water solubility (Pearlman et al., 1984) makes them less significant in aquatic toxicology. The chosen compounds are quinoline, a two-ringed structure, four three-ringed isomers (acridine, phenanthridine, benzo[f]quinoline, and benzo[h]quinoline), and two four-ringed isomers (benz[a]acridine and benz[c]acridine). With this series, the role of the number of rings and other size related parameters in inducing toxicity can be investigated, as well as the differences between isomers. Furthermore, two three-ringed metabolites were chosen: 9(10H)-acridone and 6(5H)-phenanthridinone, metabolites of acridine and phenanthridine respectively, and isomers of each other. Finally, the isomers benzo[g]quinoline-5,10-dione and benz[g]isoquinoline-5,10-dione were chosen, of which the latter was found to be highly teratogenic in crickets, while the former was not teratogenic at all (Walton et al., 1983) (Fig. 1.2).

Figure 1.2. Selected azaarenes, used in this study.
General Introduction

This study fits into a continuing effort of the Amsterdam Research Institute for Substances in Ecosystems to elucidate environmental fate and toxicity mechanisms of azaarenes, involving determination of azaarene concentrations in sediments (Kozin et al., 1997), assessment of azaarene toxicity towards algae (Van Vlaardingen et al., 1996; Wiegman et al., 1998) and invertebrates (Kraak et al., 1997a) and explaining differences in azaarene fate and toxicity by structure-activity relationships (De Voogt et al., 1991; Kraak et al., 1997b).

Transformation

Transformation of azaarenes takes place through abiotic (Kochany and Maguire, 1994) as well as biotic processes. Biotransformation is necessary to prevent a rapid build-up of toxic lipophilic compounds in the organism, and usually involves the attachment of a polar group to enhance the water solubility of the compound and therewith facilitating excretion.

In the natural environment micro-organisms play an important role in biotransformation of azaarenes (Bak and Widdel, 1986; Berry et al., 1987; Pereira et al., 1987ab; Pereira et al., 1988ab; Kuhn and Suflita, 1989; Bollag and Kaiser, 1991). Under both aerobic and anaerobic conditions, micro-organisms are able to degrade azaarenes, resulting in hydroxylated and oxygenated products. Such biodegradation, however, appears to be restricted to smaller azaarenes such as pyridine (Kuhn and Suflita, 1989; Kaiser et al., 1996), indole, quinoline and isoquinoline (Bak and Widdel, 1986; Shukla, 1986; Pereira et al., 1988b; Schwarz and Lingens, 1994; Kaiser et al., 1996), and benzoquinolines (Pereira et al., 1988b; Knezovich et al., 1990; Kaiser et al., 1996). Similar results are reported for fungi (Sutherland et al., 1994ab; Sutherland et al., 1998).

In contrast to the extensive studies on microbial degradation of azaarenes, only few data are available for (aquatic) invertebrates, algae, or vertebrates. Fish have been shown to transform quinoline into hydroxylated metabolites (Bean et al., 1985), zebra mussels can transform acridine into acridone (Kraak et al., 1997a), and also for algae acridine metabolism has been reported (Dijkman et al., 1997).

Although transformation products have been identified in sediments (Pereira et al., 1987b), the role of metabolites in the environmental effects of organic compounds is scarcely studied. Yet, it is clear that the (geno)toxic effects of a
compound cannot be assessed without considering the effects of its metabolites. As several mutagenicity and carcinogenicity studies have shown, azaarenes often show no effect until they are metabolically activated, resulting in several transformation products, including dihydrodiols and diol epoxides (e.g. Chang et al., 1984; Kumar et al., 1989; Wood et al., 1989; Warshawsky et al., 1992). Furthermore, in a toxicity study (Kraak et al., 1997a) zebra mussels demonstrated their ability to metabolise acridine to acridone, resulting in a decrease in toxicity. Using the Mutatox™ genotoxicity assay, however, acridone was determined to be much more genotoxic than its parent compound acridine (Klamer, 1996). These observations clearly stress the significance of biotransformation in the fate and (eco)toxicology of azaarenes.

Different Biological Endpoints

Any organic chemical, including azaarenes, can exhibit a narcotic effect if it is sufficiently accumulated in biological membranes (Könemann, 1981; Hermens, 1989). More specific effects, however, can occur at lower exposure concentrations (cf. Musch, 1996), especially during chronic exposure. De Maagd (1996) and Van Brummelen et al. (1998) proposed to classify four modes of action of PAHs in (aquatic) organisms: 1. apolar narcosis, 2. toxicity after photochemical activation by UV light, 3. biochemical activation leading to genotoxicity, teratogenicity and/or carcinogenicity and 4. reproductive effects. For azaarenes data on each of these modes of action are scarce and fragmentary.

LC₅₀ values for azaarenes have been determined for only a few aquatic macroinvertebrate species, among which daphnids (Southworth et al., 1978; Parkhurst et al., 1981; Newsted and Giesy, 1987), midges (Chironomus tentans; Cushman and McKamey, 1981), copepods (Diaptomus clavipes; Cooney and Gehrs, 1984) and the zebra mussel Dreissena polymorpha (Kraak et al., 1997ab). Also some results are reported on amphibians (Rana pipiens; Birge and Cassidy, 1983; Xenopus laevis; Davis et al., 1981).

Several PAHs, among which azaarenes, strongly absorb sunlight, especially in the UV region. This may lead to photochemical transformation by two different modes of reactions: photosensitization and photomodification (Veith et al., 1995; Huang et al., 1997). In photosensitization reactions, PAHs in excited state induce formation of singlet oxygen or other extremely toxic radicals (Veith
et al., 1995; Krylov et al., 1997). Photomodification structurally alters PAHs to a variety of products, mainly oxygenation products (often better soluble in water). Many of these photomodification products appeared to be more toxic than parent PAHs (Ren et al., 1994; McConkey et al., 1997; Wiegman et al., 1998).

Biochemical activation has been studied extensively in relation to mammalian and bacterial genotoxicity and carcinogenicity, but only teratogenicity studies have focussed sometimes on invertebrates (e.g. Walton, 1983), and (partly) aquatic species (e.g. Dumont et al., 1979).

Especially long-term effects such as reproduction are hardly studied (Parkhurst et al., 1981). In contrast, upon long-term exposure, particularly reproductive effects and biochemical activation leading to developmental disturbances, become more important. In absence of a proper identification and toxicity assessment for the many compounds involved, one may speculate that such compounds contribute to poorly identified effects in the environment. These effects could include the local extinction of species as well as alterations of life-cycles of invertebrates and a higher incidence of cancer in fish and man in strongly urbanised areas.

**Test Organisms**

*Chironomus riparius*

At least 15,000 different species of the dipteran family Chironomidae are distinguished, inhabiting a wide variety of freshwater lakes and streams throughout the world (Armitage et al., 1995). The life-cycle of chironomids includes an egg stage, four larval stages, a pupal stage, normally all in the aquatic environment, and an terrestrial adult stage (Fig. 1.3). After swarming and mating females deposit egg masses at the water surface, usually attached to some kind of substrate, either natural such as plants or artificial such as bridges. First instar larvae are principally planktonic, but in most species second to fourth instars inhabit the upper layer of the sediment, in which they build protective tubes from small particles (Armitage et al., 1995).

In this study the species *Chironomus riparius* has been used. The larvae of this species prefer eutrophic and organic enriched waters (Armitage et al., 1995) and
are also frequently found in rivers or canals affected by effluents of sewage treatment plants (Köhn and Frank, 1980; Davies and Hawkes, 1981). C. riparius is an opportunistic species: it is among the first macro-invertebrates to colonise streams during temporary periods of eutrophication or organic enrichment (Gower and Buckland, 1978). This opportunistic behaviour is facilitated by its multivoltine life cycle, on our latitude at least three generations a year in organically enriched rivers (Gower and Buckland, 1978). However, in a prairie pond in Canada, univoltine life cycles were observed, due to a shorter ice-free period (six months) and relatively low food abundances (Rasmussen, 1984). At 20 °C a complete life cycle can be completed within three weeks, but at 12 °C this takes twice as long (Gower and Buckland, 1978). Due to this short life cycle C. riparius can easily be cultured in the laboratory (Credland, 1973).

![Figure 1.3. Life cycle of Chironomidae displaying the egg stage, the four larval instars, the pupal stage and the terrestrial imago (adopted from Timmermans, 1991).](image)

Because of its world-wide distribution and because it is easy to handle in the laboratory, many toxicological studies have been performed with C. riparius
(637 items in the AQUIRE toxicological database; http://www.epa.gov/ecotox; July, 1999). This enables the comparison of results from this study with literature data. The midge has also been used in ecological studies (Heinis, 1993; Van de Bund, 1994) and it has proven to be useful for monitoring purposes (Grootelaar et al., 1995; Stuijfzand, 1999). Adaptation strategies of \textit{C. riparius} to metals have also been studied extensively (Postma, 1995; Groenendijk, 1999).

In the present study, a laboratory culture was used, which has been maintained at the Department of Aquatic Ecology & Ecotoxicology since 1986 at 20 °C (16:7 h light:dark regime, separated by 0.5 h twilight), originating from larvae from a small experimental pond of the University of Amsterdam. To minimise the risk of inbreeding, egg masses were regularly exchanged with other Dutch laboratory cultures of \textit{C. riparius}. In addition, the culture was constantly maintained at a large population size.

\textit{Fluctuating asymmetry}

Fluctuating asymmetry (FA) is defined as the occurrence of random differences between the phenotypic values of characters on the left and right sides of a normally bilaterally symmetrical individual organism (Van Valen, 1962). It has frequently been suggested that FA can be a reliable index of genetic or environmental health and of the effects of different types of stress (Leary and Allendorf, 1989; Parsons, 1992). In chironomids FA has been proven to be a good measure for environmental stress, such as metal contamination (Janssens de Bisthoven et al., 1998; Groenendijk, 1999), insecticide contaminants (Clarke et al., 1995), and co-occurring toxicants (PAHs, PCBs and metals; Van Urk et al., 1992). Research on the chironomid \textit{C. plumosus} has shown that reproductive fitness can also be influenced by FA: females of this species prefer to mate with more symmetric males (McLachlan and Cant, 1995).

For a reliable demonstration of FA, other forms of asymmetry normally occurring in certain animal populations, should be excluded (Palmer and Strobeck, 1986; Palmer, 1994; Swaddle et al., 1994). The first of these is antisymmetry, which is due to negative interaction between sides. This type of asymmetry is found for example in fiddler crabs (\textit{Uca} spp), where the large signalling claw of the males occurs at an equal frequency on the left and right sides in a population. Another form of asymmetry that can seriously interfere with FA is directional asymmetry. This form of normal development is for
example occurring in humans where the right gonads are normally larger than those on the left (cf. Leary and Allendorf, 1989).

In the present thesis, FA was used as a measure for developmental disturbances in *C. riparius* larvae exposed to azaarenes (Chapter 6). The character for FA analysis chosen was the number of teeth on the comb-like pecten epipharyngis (Fig. 1.4), one of the larval mouthparts, situated at the anterior part of the larval head capsule.

**Figure 1.4.** Pecten epipharyngis of *Chironomus riparius* situated at the anterior part of the larval head capsule. A: normal pecten epipharyngis; B: deformed pecten epipharyngis with fused teeth (adapted from Vermeulen, 1995).

**The Mutatox™ test**

To evaluate the genotoxic potential of azaarenes, the Mutatox™ test was used (Chapter 5). In this genotoxicity test a dark variety of the normally luminescent bacteria *Vibrio fisheri* is used. Under influence of genotoxicants the luminescent ability can be restored (Ulitzur, 1986; Johnson, 1992). This restoration can theoretically be achieved by three independent events (Ulitzur, 1986): (1) blocking the formation of the repressor, i.e. altering its or the operator site’s structure, (2) inactivating the repressor of the luminescence system, and (3) changing the physical configuration of the DNA, thus allowing unpressed
transcription of the luciferase operon. Blocking of the repressor is expected from direct mutagens (Ulitzur, 1986). Different DNA-damaging agents and DNA synthesis inhibitors seem to act through their ability to trigger the "SOS functions" that involve the inactivation of the luminescence system's repressor (Weiser et al., 1981). Finally, DNA-intercalating agents act via the most potent and rapid way of restoring the luminescence by changing the physical configuration of the DNA (Ulitzur, 1986). Direct and indirect genotoxicity can be distinguished by incubation with or without a S9-enzyme-preparation, but in this thesis (Chapter 5), only direct genotoxicity is presented.

![Graph showing LOEC determination](image)

Figure 1.5. Example of the determination of the LOEC for a test compound.

To quantify the genotoxicity of compounds, a Lowest Observed Effect Concentration (LOEC) can be determined. This concentration is defined as the concentration at which the light response is 4 times that of the control response (Fig. 1.5).

The fact that three different kinds of genotoxic activity can be scored within one test, makes this test favourable above other genotoxicity tests, such as the SOS Chromotest and the often used Ames test. Furthermore, in general the Mutatox™ test was shown to be more sensitive than the SOS Chromotest (Legault et al., 1994), and also mutagenicity in the Ames test was well predicted.
(93 % of the cases tested) by the Mutatox™ test (Legault et al., 1994). In addition, the Mutatox™ assay is more rapid to perform and, as a result, costs less (Johnson, 1992).

**Outline of the Thesis**

The present study analyses the different aspects of azaarene toxicity by evaluating the different kinds of biological effects that these compounds can exhibit. Biotransformation and isomerism receive special attention. Structure-activity relationships are used to examine the toxicological mechanisms involved.

In Chapter 2 and 3 of this thesis metabolism of azaarenes is studied in a variety of aquatic organisms. Chapter 2 focuses on identification and quantification of 9(10H)-acridone and 6(5H)-phenanthridinone as major metabolites of, respectively, acridine and phenanthridine in experiments with several invertebrates and algae. Chapter 3 compares the capacity to transform phenanthridine between an invertebrate (the midge *Chironomus riparius*) and a fish (*Cyprinus carpio*).

Chapters 4 to 6 focus on different aspects of the toxicity of azaarenes. Each of these chapters focuses on a different (eco)toxicological endpoint: acute toxicity (narcosis), genotoxicity and life cycle parameters.

In Chapter 4 96 h LC$_{50}$ values are estimated for midge larvae (*Chironomus riparius*) exposed to seven azaarenes: quinoline, acridine, phenanthridine, benzo[f]quinoline, benzo[h]quinoline, benz[a]acridine, and benz[c]acridine (Fig. 1.2), which include four three-ringed isomers and two four-ringed isomers. In addition, relationships between LC$_{50}$ and molecular descriptors were sought to gain insight into the mechanism of acute toxicity.

In Chapter 5 the Mutatox™ test is used to evaluate the genotoxic potential of both the non-substituted azaarenes used in Chapter 4 and the two metabolites acridone and phenanthridone, which enables comparison between isomers (including the metabolite pair) as well as the role of metabolism. Again relationships are sought with molecular descriptors and, furthermore, a comparison is made between the genotoxicity and the acute toxicity as reported in Chapter 4.
Chapter 6 completes the section on toxicity with a study on chronic effects of azaarenes on the midge *Chironomus riparius*, using developmental disturbances and day of emergence as parameters. Six three-ringed azaarenes were selected: acridine and phenanthridine, together with their metabolites acridone and phenanthridone, and in addition the isomers benzo[g]quinoline-5,10-dione and benz[g]isoquinoline-5,10-dione. Thus, comparisons could be made between isomer pairs and between parent-metabolite pairs. Finally, a comparison was made between the chronic toxicity data and the toxicity and genotoxicity data from Chapters 4 and 5.

In the last part of the thesis (Chapter 7), a molecular mechanics approach is used to study the interactions of all eleven azaarene molecules (Fig. 1.2) with the phospholipid bilayer of a biological model membrane. After placing each azaarene molecule separately in a bilayer model, constructed from sixteen 1,2-dimyristoyl-sn-glycero-3-phosphorylcholine molecules, interacting energies were calculated and relationships between these energies and (geno)toxicity parameters were sought.

Finally, in Chapter 8, overall conclusions are summarised and implications for risk assessment are outlined.

References


